NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
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Submitted by

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Project Director

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United States Army Medical Research and Development Command
(Walter Reed Army Institute of Research)

and

Louisiana State University Medical Center
Neuroscience Center of Excellence

Volume 7 of 8

Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury

Project Directors:
Prescott Deininger, Ph.D.
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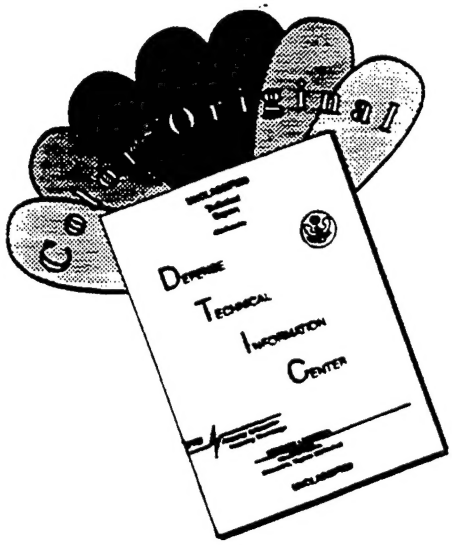
DISCLAIMER NOTICE



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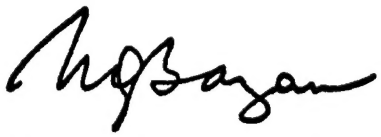
This Technical Report covers the progress made in the second year of this Cooperative Agreement in one project of the original proposal. We hope that this format of the report will facilitate its handling. The table of contents for all the projects has been included in each volume.



Nicolas G. Bazan, M.D., Ph.D.
Director, LSU Neuroscience Center
Program Director, USAMRDC Cooperative Agreement

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INTRODUCTION

Head injury triggers a time sequence of biochemical changes that can result in either cell death or wound healing and recovery. Second messengers are released as a consequence of cell signal transduction activation by trauma. Some of these messengers may target the nucleus and activate gene expression (Bazan, 1994). As a result, new signal molecules such as cytokines and peptide growth factors can be generated, released from the cells, and can interact with receptors in other cells. The cellular responses generated can have positive (repair) or negative (death) consequences.

This project aims to contribute to a better understanding of the role of specific growth factors, the differentiation of growth factors, neurotrophic factors and cytokines in development, and wound healing in the central nervous system (CNS) and eye. We had two goals in our original proposal. The first was to use our dominant negative mutants for PDGF to specifically lower neuronal PDGF expression, in order to determine if either development or the response to wound models was altered. These experiments are well under way. Our second goal was to survey gene expression, using several injury models, for a number of genes thought to participate in the repair process after brain injury.

BODY

Previous work. In the first year a great deal of effort was put into making a genetic construct that expresses the dominant negative mutant in a neural-specific expression pattern in transgenic mice.

Objectives, year 2. During the last year, DNX injected that construct into mice and generated eight independent lines of transgenic mice carrying this construct. PCR analysis confirmed the integration of the transgene in these mice. We then bred the animals to generate homozygotes, and analyzed these for expression of the transgene in mouse brain using RT-PCR. We were able to demonstrate that the transgene is expressed at a fairly high level in a brain-specific manner.

The objectives of the second year were to study: **I- The role of PDGF in brain development.** The objective of this study was to determine the roles of PDGF in the brain by generating and studying mice deficient in brain PDGF A and B forms. We chose to use a dominant negative mutant cDNA of PDGF, which has been shown to bind to and inactivate both PDGF A and B forms by heterodimerization. **II- Gene expression following brain injury.** The objective of this study was to assay for changes in the expression of genes involved in neural growth and differentiation as a function of wound healing. We used the Chalifour procedure to assay for changes in panels of brain cortex monas.

Methods

Cryogenic Injury Protocol.

The animal model of vasogenic brain injury was generated by the placement of a liquid nitrogen-cooled probe, (at a b. p. of -195.79°C), against the exposed skull of the rat for one minute. The cold probe consisted of a brass rod 9 mm in diameter and 25 mm in length, with a concave tip that fit against the curved surface of the skull. The probe was attached to a larger brass rod 16 mm diameter and 55 mm in length which acted as a heat sink and was bound to a 30 cm stainless steel handle. The handle was insulated toward its end with Delrin^{R} to avoid injuries to the operator during the experimental procedures. This probe was immersed in liquid nitrogen until the moment it was to be applied to the skull surface.

Wistar rats weighing 250-275 g were subjected to ether anesthesia and their skulls were exposed by an incision along the scalp to the midline. The cold probe was then rapidly pressed onto the right fronto-parietal region of the skull for a period of one minute and the animals were

sacrificed after injury at time intervals of 1 hr, 12 hrs, 1 day, 3 days, 1 week, and 4 weeks. A control group of 3 animals was alloted. The animals underwent the same procedure except for probe application. Brain tissue was then rapidly dissected on an ice cold dissection board at the specified times.

Analysis of gene expression patterns.

Double stranded radiolabeled cDNA was synthesized from rat cortex RNAs isolated at various time points following brain injury. Panels of nitrocellulose filter fixed cDNA clones were then screened according to the method of Chalifour et al. (1994). Modifications included the use of 50 μg of RNA, 2000u reverse transcriptase, 120 μCi ^{32}P-dCTP, and 2u of klenow per sample. Nitrocellulose filters were hybridized to 10^6 cpm/ml of brain cDNA in 10 ml of hybridization solution.

To our surprise, even though the dominant negative expression should have greatly decreased PDGF production from neuronal cells, there was no detectable developmental phenotype in these animals. We hypothesize that even with a significant decrease in production of PDGF from the neurons, PDGF produced by glial cells, or even transported across the blood-brain barrier, may compensate for its low production of brain tissue. In studies based on cultured cells, PDGF has been hypothesized to have a pivotal role in brain glial cell development and differentiation. Our finding suggests that, if this is so, it is a process that has a great deal of latitude in terms of the specific level and distribution of PDGF expression. Alternatively, upon altering PDGF, there may be some other compensatory change to maintain normal development.

II. *Gene expression following brain injury.*

Brain tissue was collected at various time intervals after injury. These include 1 hr, 12 hrs, 1 day, 3 days, 1 week, and 4 weeks. The method of Chalifour was then used to simultaneously assay relative gene expression levels from a broad bank of genes. These genes are transferrin, transferrin receptor, neurofilament, proteolipid protein (PLP), EGF-receptor, NGF-beta, Rb (included in **Figure 3**), pUC, DHFR, IGF1 receptor, IGF1, acidic FGF, somatostatin, PDGF alpha receptor, catalase (included in **Figure 4**), BSSK, GFAP, superoxide dismutase (SOD), renin, p53, myelin basic protein (MBP), tubulin, G6PD (included in **Figure 5**), BDNF, CNTF, rRNA (included in **Figure 6**), angiotensin, IL-6, v-fos, laminin, basic FGF, TNF alpha, PDGF- A chain, insulin (included in **Figure 7**), fibronectin, IL-2, TGF beta 1 and beta 2, actin, TRK-B, Tyrosine kinase, POMC, insulin receptor (included in **Figure 8**), PDGF-B2, TH, c-jun, EGF, c-fos, CSF, and PDGF-B (included in **Figure 9**). The approach was to bind clones for each of these genes or cDNA to multiple nitrocellulose strips. A radiolabelled cDNA probe was then prepared from the mRNAs from each of the brain injury time points. Identical nitrocellulose strips were then hybridized to the probe from each mRNA time point. Those specific mRNAs expressed to higher levels will produce higher signals, allowing estimation of the expression of the different genes relative to one another. Furthermore, by comparing the patterns of gene expression during the different injury time intervals, we can assess the changes immediately after injury and during the repair process.

We have measured high levels of expression of a number of genes. The increase observed in genes such as IGF1, CSF, TGF beta 2, and transferrin receptor may be associated with cell

growth. Several other genes decreased their expression. We are repeating these experiments to increase sensitivity and reliability, but are very pleased with the results of the procedure so far.

7-SUMMARY AND CONCLUSIONS

I. Although all our controls suggest that we have obtained appropriate expression of the dominant negative PDGF construct in the brain, we have observed no detectable phenotype. This suggests the possibility of redundancy or backup systems in the role of PDGF in the brain. However, the animals in the wound models may demonstrate that there is still a need of PDGF in critical situations. These data will ultimately provide evidence regarding the effect of decreasing PDGF, i.e. whether it is likely to have either positive or negative effects on therapy.

II. Our initial data show the relative steady-state levels of various polyadenilated mRNAs in the brain. Transferrin receptor, IGF-1, α-FGF, renin, MBP, α-tubulin, actin, tyrosine kinase, IL-2, laminin, and angiotensinogen are all highly represented mRNAs. In contrast, many mRNAs, including PLP, PDGF A and B, TGF β1 and catalase, are expressed at very low levels. Some mRNAs appear to be induced over the four week period following injury (transferrin receptor, MBP, and IGF-1.) Alternatively, and as one would predict, other mRNAs appear to be repressed, to undergo transient changes, or to be expressed non-differentially.

Research plan for the upcoming year

- Even though our transgenic mice expressing the dominant negative PDGF mutant did not show developmental anomalies, there is extensive evidence for PDGF playing a role in wound healing. Accordingly, we will now test these animals in injury models (i. e. ischemia/reperfusion, cryogenic injury, retinal light damage) to determine whether altered PDGF levels affect wound healing in these systems.
- Our major effort in the coming year will be a further characterization of gene expression in the brain wound models. We will repeat several more times the scans of changes in gene expression in the cerebral cortex following cryogenic injury. Similar experiments will be conducted using rat hippocampus mRNAs.
- We will include other genes, as deemed appropriate, and will carry out the experiments to make them more sensitive and to demonstrate reproducibility. In key genes whose expression levels are

not high enough to provide reliable data using our current method, we will utilize direct Northern blots with individual probes to increase the sensitivity.

8- REFERENCES

Bazan, N. G. (1994) Signals, messengers and genes in cerebral ischemia: Novel sites for neuroprotection. In: Krieglstein, J. and Oberpichler, -Swenk, H (eds) Pharmachology of cerebral ischemia, pp. 1-13. Wissenschaftl, Stuttgart.

Chalifour, L. E., Fahmy, R., Holder, E. L., Hutchinson, E. W., Osterland, C. K., Schipper, H. M., Wang, E. (1994) Analytical Biochem. 216, 299-304.

Figure 3. RNA expression as a function of time following injury. Expression was determined by dot blot analysis using the Chalifour method. R, receptor; PLP, proteolipid protein; EGF, epidermal growth factor; NGF, nerve growth factor; Rb, retinoblastoma gene product.

Figure 4. RNA expression as a function of time following injury. Expression was determined by dot blot analysis using the Chalifour method. pUC , pUC control plasmid; DHFR, dihydrofolate reductase; IGFI-R, insulin-like growth factor receptor, IGFI, insulin-like growth factor, aFGF, acidic fibroblast growth factor; PDGF-A-R, platelet-derived growth factor A chain receptor.

Figure 5. RNA expression as a function of time following injury. Expression was determined by dot blot analysis using the Chalifour method. BSSK, Bluescript control plasmid; GFAP, glial fibrillary acidic protein; SOD, superoxide dismutase; p53, p53 tumor supressor protein; MBP, myelin basic protein; G6PD, glucose-6-phosphate dehydrogenase.

Figure 6. RNA expression as a function of time following injury. Expression was determined by dot blot analysis using the Chalifour method. TGF-B2, transforming growth factor β2; TH, tyrosine hydroxylase; PDGF-B-R, platelet-derived growth factor B chain receptor; EGF, epidermal growth factor; CSF, colony-stimulating factor; PDGF-B, platelet-derived growth factor B chain.

Figure 7. RNA expression as a function of time following injury. Expression was determined by dot blot analysis using the Chalifour method. BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; rRNA, 18s ribosomal RNA.

Figure 8. RNA expression as a function of time following injury. Expression was determined by dot blot analysis using the Chalifour method. IL-6, interleukin 6; bFGF, basic fibroblast growth factor, PDGF-A, platelet-derived growth factor A chain.

Figure 9. RNA expression as a function of time following injury. Expression was determined by dot blot analysis using the Chalifour method. IL-2, interleukin 2; TGF-B1, transforming growth factor β1; TRK-B, nerve growth factor receptor; POMC, proopiomelanocortin; insulin-R, insulin receptor.

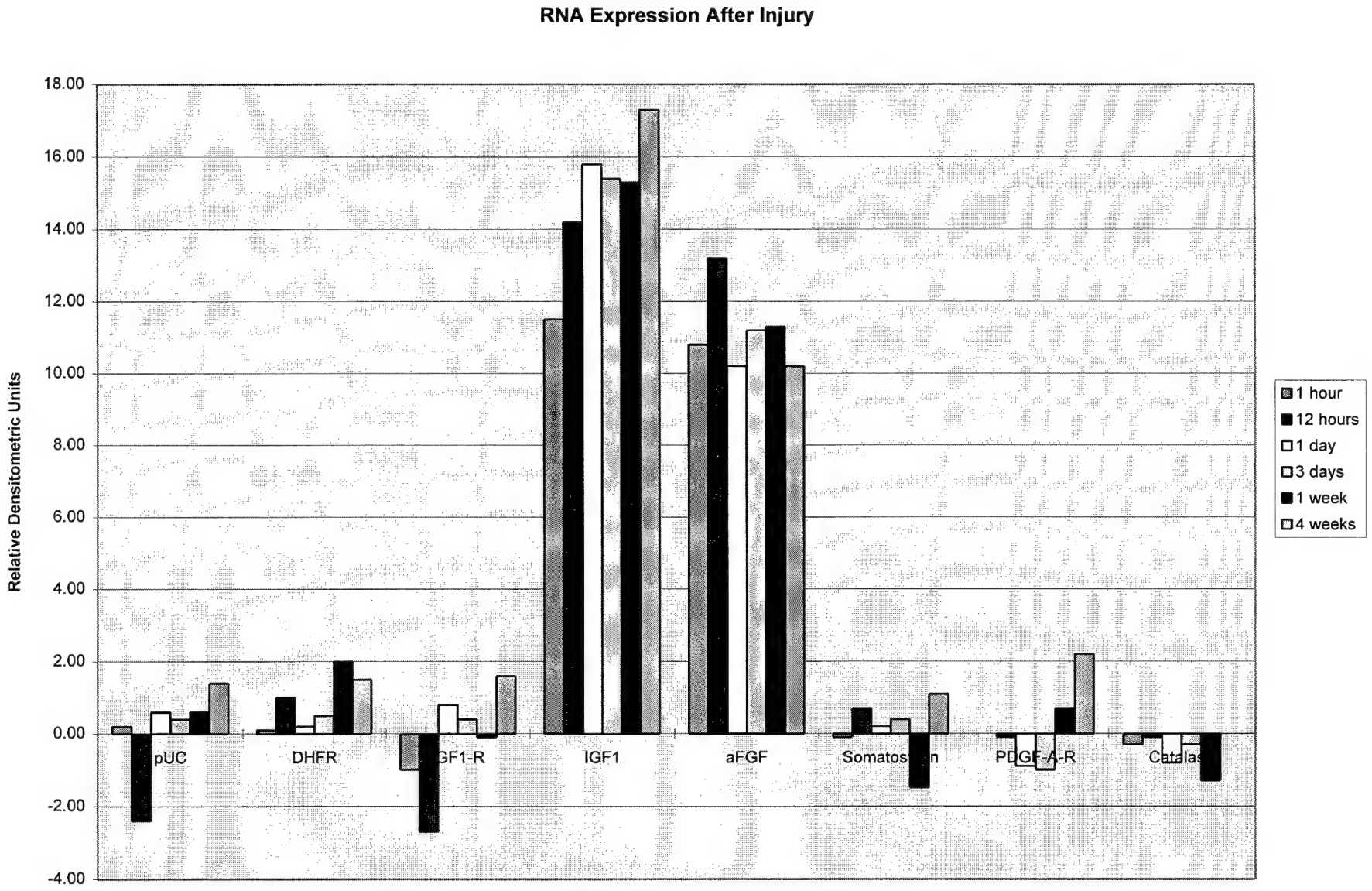

RNA Expression After Injury
Relative Densitometric Units
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
-4.00
pUC
DHFR
GF1-R
IGF1
aFGF
Somatos n
PDGF-A-R
Catalas
1 hour
12 hours
1 day
3 days
1 week
4 weeks

Figure 4

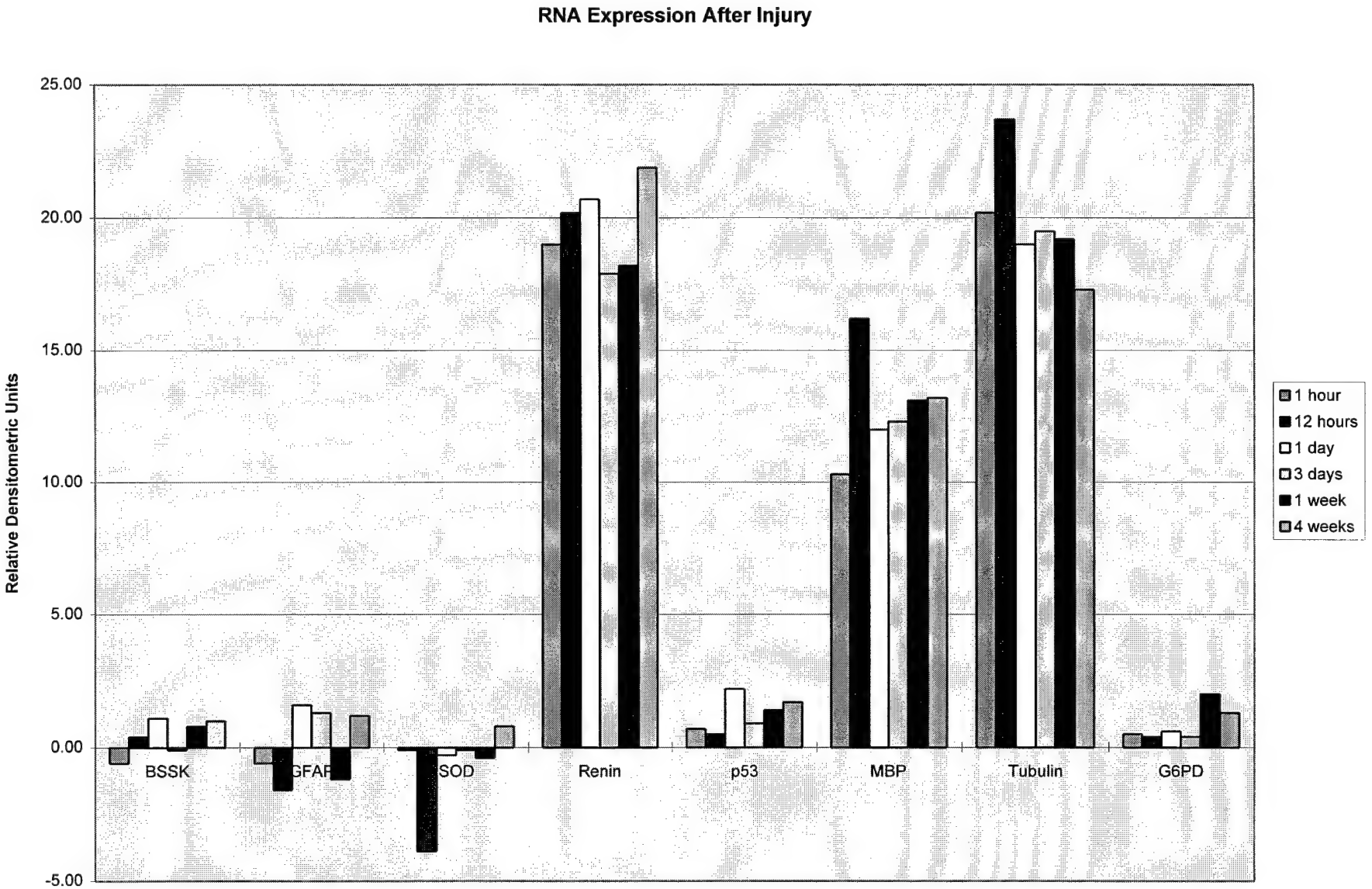

RNA Expression After Injury
Relative Densitometric Units
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
BSSK
GFAP
SOD
Renin
p53
MBP
Tubulin
G6PD
1 hour
12 hours
1 day
3 days
1 week
4 weeks

Figure 5

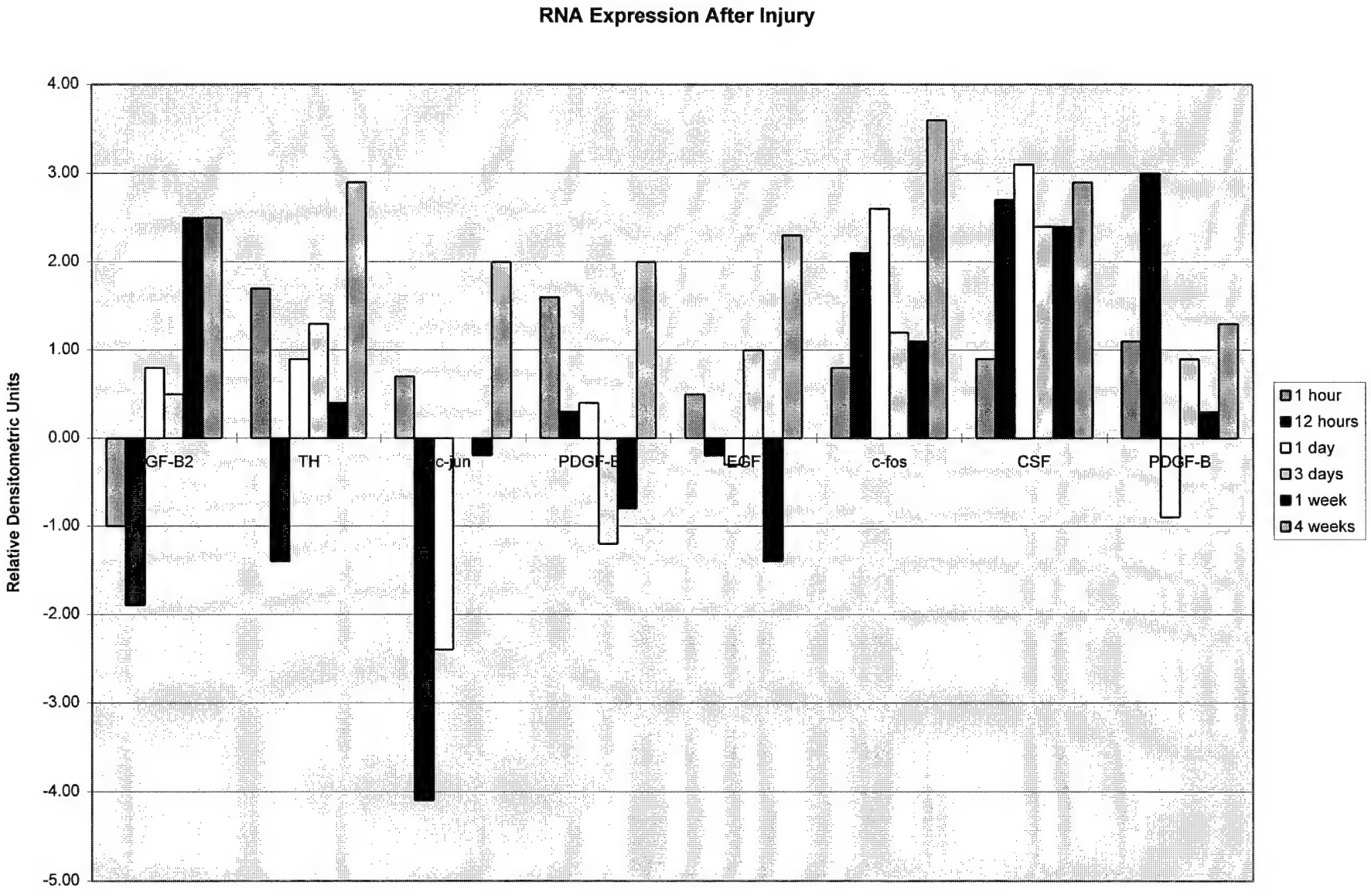

RNA Expression After Injury
Relative Densitometric Units
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
GF-B2
TH
c-jun
PDGF-B
EGF
c-fos
CSF
PDGF-B
1 hour
12 hours
1 day
3 days
1 week
4 weeks

Figure 6

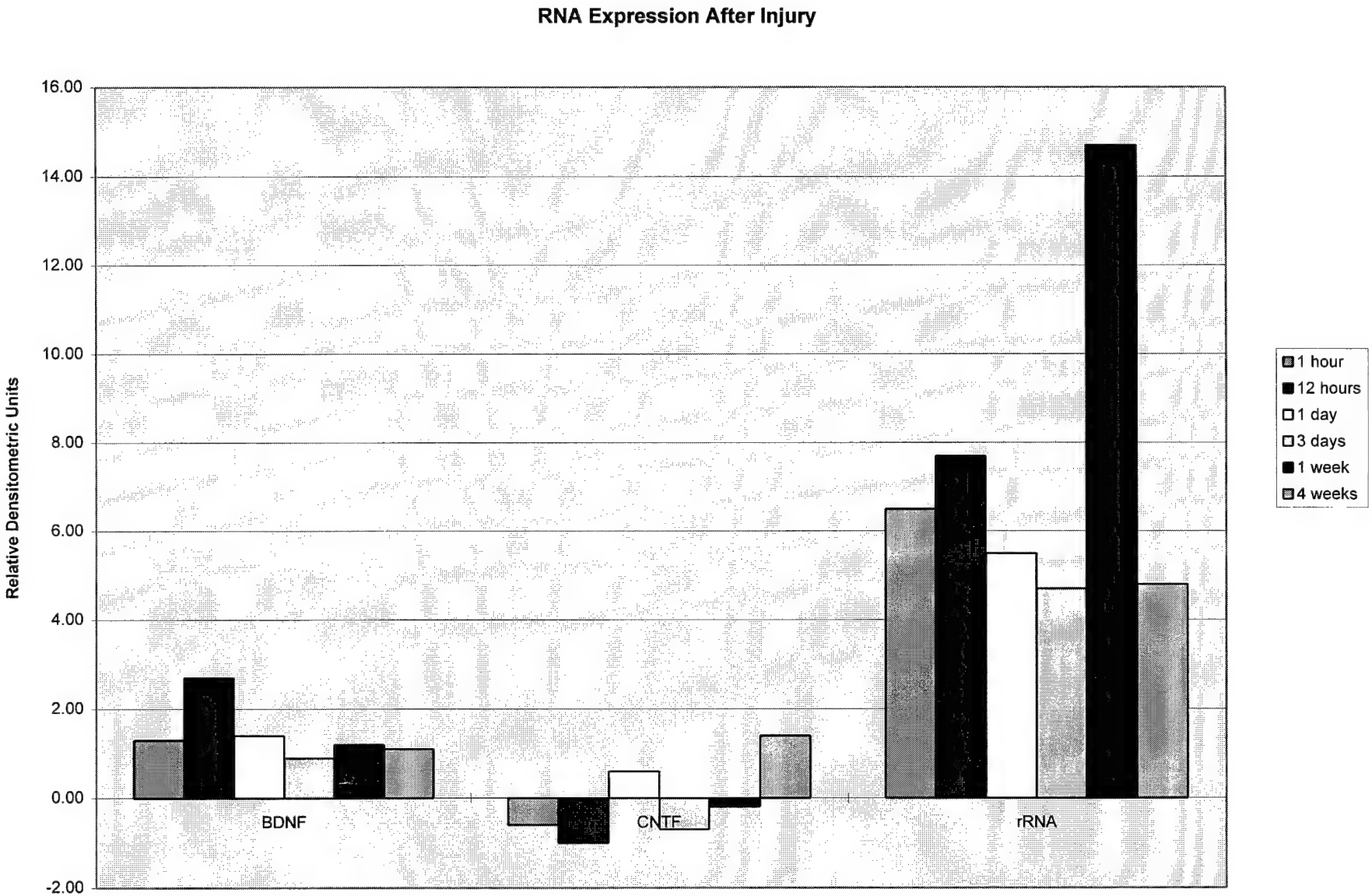

RNA Expression After Injury
Relative Densitometric Units
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
BDNF
CNTF
rRNA
1 hour
12 hours
1 day
3 days
1 week
4 weeks

Figure 7

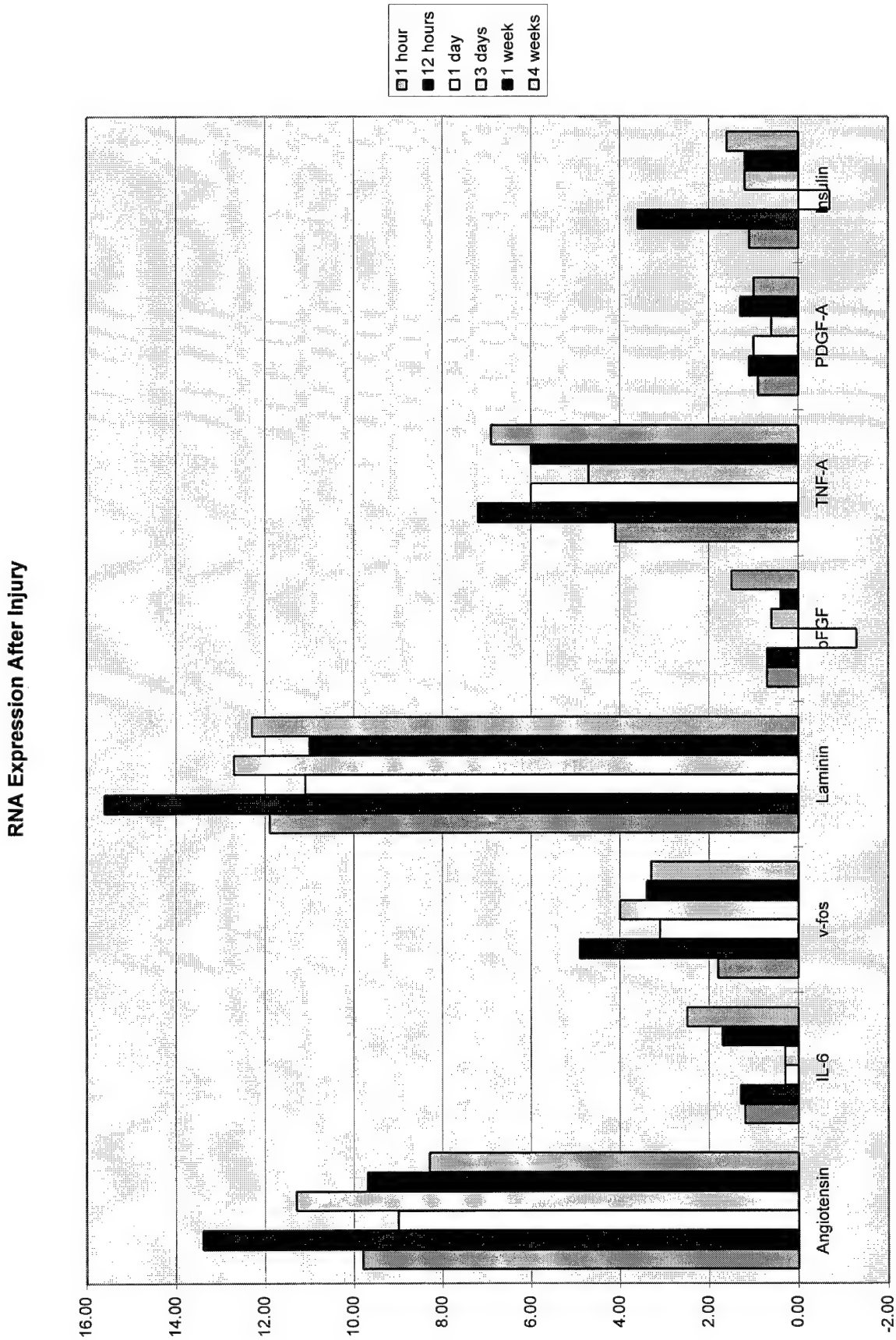

RNA Expression After Injury
1 hour
12 hours
1 day
3 days
1 week
4 weeks
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
Relative Densitometric Units
Angiotensin
IL-6
v-fos
Laminin
bFGF
TNF-A
PDGF-A
Insulin

Figure 8

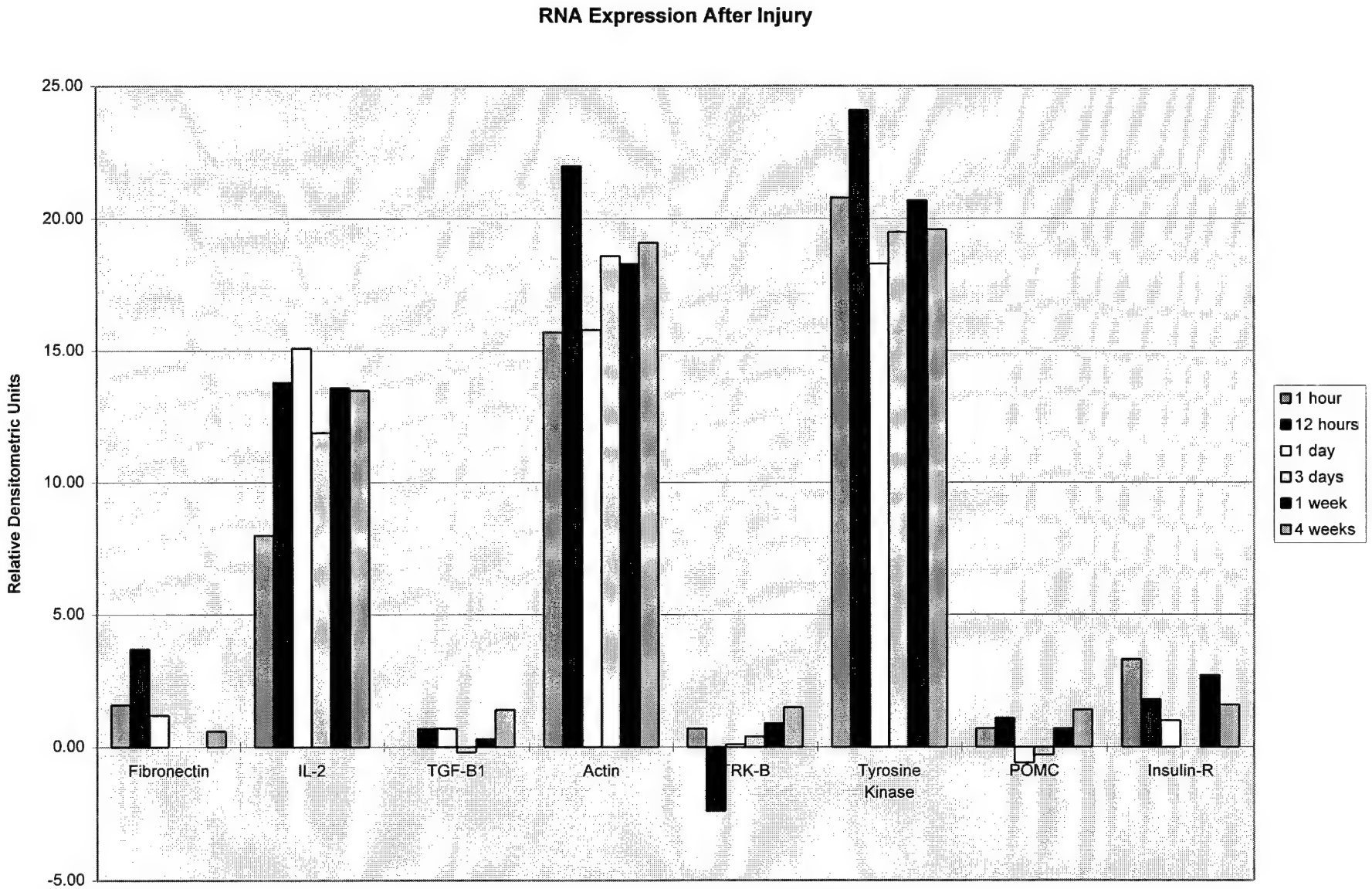

RNA Expression After Injury
Relative Densitometric Units
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
Fibronectin
IL-2
TGF-B1
Actin
TRK-B
Tyrosine Kinase
POMC
Insulin-R
1 hour
12 hours
1 day
3 days
1 week
4 weeks

Figure 9

ALTON OCHSNER
MEDICAL FOUNDATION
1516 Jefferson Highway
New Orleans, Louisiana 70121
Phone: 504 838-3000
Fax: 504 837-0977
Cable Address: OCHSCLINIC

Ochsner

Rick Huntress, Transgenic Services Coordinator
DNX Corporate Headquarters
303B College Road East
Princeton, New Jersey 08540

February 22, 1994

Dear Rick:

We have assembled a plasmid within which an expression construct for a PDGF dominant negative mutant (1) exists. The promoter is from the neuron-specific enolase gene and should direct expression to neuronal tissue (2). In previous transgenic mice, this promoter lead to specific expression of a regulated β-galactosidase gene in CNS neurons (2). Since the expression construct is assembled in the plasmid, pMT2, the mRNA is expressed as a fusion construct of PDGF-DHFR (mouse dihydrofolate reductase cDNA) (3). We have constructed our primers to the DHFR portion of the transgene. The primers can amplify the transgene to produce a 300 bp product. The primers can also amplify endogenous genomic DNA but will produce a >6 kb fragment. We have had no problems with amplification of the endogenous genomic DNA (no visible high MW band and no obvious competition with the transgene for primer binding). The transgene fragment has been prepared according to your specifications and has been purified free of most plasmid sequences by digestion with Bam HI and Ssp I. The fragment is 4817 bp in length and a circular map is provided. The gene will produce no infectious condition which could be harmful to other animals, humans or the environment and experimentation involving the gene does not require containment conditions greater than those required under NIH RAC BL-2 standards.

We are providing you with purified transgene (200 ng/ul) and PCR primers (20uM). In addition, we are providing photographs and related information regarding quantification and PCR amplification of the fragment. Please contact me if I can provide additional information or if I can answer any questions. Thanks very much, Rick.

1) Mercola, M., Deininger, P. L., Shamah, S. M., Porter, J., Wang, C. and Stiles, C. D. 1990. Dominant-negative mutants of a platelet-derived growth factor gene. Genes and Devel. 4:2333-2341.

2) Forss-Petter, S., Danielson, P. E., Catsicas, S., Battenberg, E., Price, J., Nerenberg, M., and Sutcliffe, J. G. 1990. Transgenic mice expressing b-galactosidase in mature neurons under neuron-specific enolase promoter control. Neuron 5:187-197.

3) In Molecular Cloning: A Laboratory Manual, eds, Sambrook, Fritsch, Maniatis, Cold Spring Harbor Laboratory Press, 1989.

Regards,

Julia L. Cook
Division of Research
Phone: 504-842-3316
FAX: 504-842-3381

DNX

DNA FRAGMENT INFORMATION

Investigator Name: Julia L. Cook

Institution: Ochsner Medical Foundation

Address: Molecular Genetics Research Division

1516 Jefferson Hwy LA 70121

Phone#: (504) 842-3316 Fax #: (504) 8 -3381

DNA Fragment Name: NSE/PDGFβ308 (NSE/pMT2ΔDRa/PDGFβ3C8/LUC)

Total DNA Fragment Length: 4817bp

DNA Fragment Purified By (check one):

ELUTIP-D (Schleicher & Schuell) XX

Qiagen Column (Qiagen, Inc.) ____

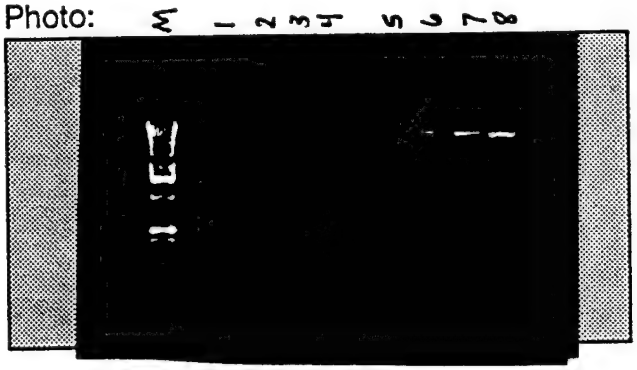
Other (Prior consultation with DNX required) ____

Please describe: ____

DNA Fragment Stock Concentration: 200 ng/μl

DNA fragment should be dissolved in 10mM Tris/0.25mM EDTA, pH 7.5. A photo of the fragment on an agarose gel with the appropriate size and quantitative markers is required.

Photo:



Comments:

Marker - 1 kb ladder

1-4 = Control, 10-50ng respectively.

5-8 = 1, 2, 4 and 5 μl of dilute transgene.

1 μl dilute transgene (1/5) = 40ng

40ng · 5 (dil'n factor) = 200ng/μl (conc. of transgene)

NTDF

PCR ANALYSIS

If PCR analysis is to be used to identify transgenic mice, NTDF requires appropriate oligonucleotides and a specification of proven conditions for the reaction. If necessary, NTDF will provide endogenous mouse DNA for evaluating oligonucleotide performance. Completion of the following information is required. Include photographs of the primer and photographs of the requested controls (originals or quality copies).

If these criteria are not met, all remaining DNA materials will be returned to the investigator with explanation and documentation and NTDF shall not be obligated to proceed further.

5' Primer name: Amp 6
Length: 25 nt
Molar Concentration of Stock: 20 uM

3' Primer name: Amp 5
Length: 25 nt
Molar Concentration of Stock: 20 uM

Reaction Conditions

All primers must be tested prior to submission. Controls should include:

1. Normal mouse DNA (NM)
2. NM + 1 gene copy/cell equivalent of the DNA fragment
3. NM + 5 gene copy/cell equivalent of the DNA fragment
4. NM + 10 gene copy/cell equivalent of the DNA fragment
5. 1 gene copy/cell equivalent of the DNA fragment alone
6. 5 gene copy/cell equivalent of the DNA fragment alone
7. DNA from a donor of same species as the DNA fragment (if applicable)

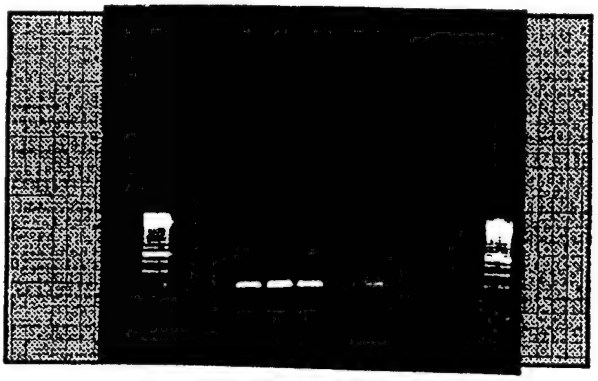
Primer Concentration in Reaction: 0.5 uM
Template concentration in the Reaction: 1-10 pg/ml

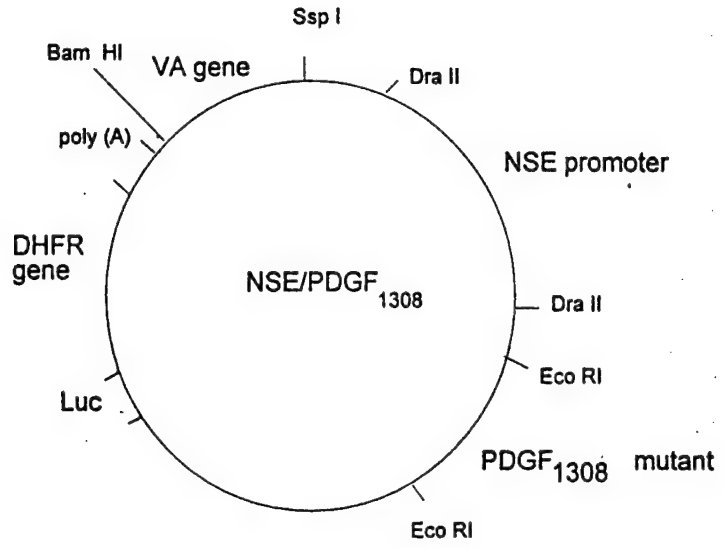
Denaturing Temp: 94°C
Annealing Temp: 55°C
Extension Temp: 72°C

Denaturing Time: 1'5"
Annealing Time: 1'5"
Extension Time: 2'5"

# of Cycles: 35

Please include a copy of the results (original or quality copy).





Luc is 540 bp XbaI/EcoRI frgmt from pXP2 (end-filled) ligated into AflII site of pMT2.

NSE/PDGF$_{1308}$ = (NSE/pMT2ΔDra/PDGF$_{1308}$/luc) is a total of 7301 bp.

The transgenic frgmt:

Cut NSE/PDGF$_{1308}$ with BamHI/SspI to generate a 4817 bp transg. frgment.

ANIMAL ID (#, sex, color)	EAR TAG #	GENOTYPE	BIRTH
3869 FEMALE BLACK	6869	-	06/26/94
3870 FEMALE AGOUTI	6870	+	06/26/94
3876 FEMALE AGOUTI	6871	+	06/26/94
3877 FEMALE AGOUTI	6872	+	06/26/94
3884 FEMALE AGOUTI	6873	+	06/26/94
3885 FEMALE AGOUTI	6874	+	06/26/94
3891 MALE AGOUTI	6875	+	06/26/94
3894 MALE ALBINO	6876	+	06/26/94
3895 MALE AGOUTI	6877	-	06/26/94
3896 MALE AGOUTI	6878	+	06/26/94
3902 MALE BLACK	6879	+	06/26/94
3906 MALE ALBINO	6880	+	06/26/94

RETLD-DO.WPD

October 11, 1995

TITLE: NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR.

CHAPTER: Role of growth factors and cell signaling in the response of brain and retina to injury

PATHOPHYSIOLOGICAL EVENTS TRIGGERED DURING LIGHT-INDUCED DAMAGE TO THE RETINA.

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INTRODUCTION

Retinal light damage involves a complex series of events modulated by environmental light history, genetic characteristics, age, and the type of light used, as well as the exposure paradigm employed. Whether the trigger for damage is localized in the outer segment, the mitochondria, or another subcellular organelle, the entire visual cell is secondarily involved at an early time during light exposure. Whatever the secondary light damage turns out to be, it occurs within hours of light onset and appears to precede and initiate cellular death.

While much has been learned from biochemical studies of the retina (Bazan and Reddy, 1985), the exact processes leading to retinal cell death are not yet clear. There are two different types of cell death: necrosis, which is triggered by injury, and leads to disruption of cell membranes and organelle swelling; and apoptosis, also known as a programmed cell death, which is regulated by physiological stimuli and is involved in cell death during development and growth of normal tissue. Apoptosis can also result from cell injury (Gerschenson and Rotello, 1992).

In the retina, apoptosis has been observed during development (Young, 1984; Penfold and Pravis, 1986; Portera-Cailliau et al., 1994), and has also been shown in mouse and rat models of inherited retinal degenerations (Chang et al., 1993; Lolley et al., 1994; Papermaster and Nir, 1994; Tso et al., 1994; Papermaster and Windle, 1995). In post-mitotic cells, apoptosis could be induced by a number of exogenous factors, including growth factor removal, irradiation, and anti-cancer drugs. Finally, light-induced retinal damage has been shown to cause apoptotic nuclear damage in a rat model (Shahinfar et al.,1991; Szcesny et al.,1994). Also, UV cones in the retina of the Atlantic salmon die as a result of apoptosis (Kunz et al., 1994).

Currently, we know little about the light damage mediator(s) generated early on in the cascade of biochemical events leading to cellular death. The biologically active phospholipid platelet activating factor (1-*O*-alkyl-*sn*-glycero-3-phosphocholine, PAF), a phospholipase A_2 (PLA_2) product, has been located in the retina (Bussolino et al., 1986) and has been implicated as one of the mediators involved in light damage induced retinal degeneration (Remé et al., 1992). PAF plays different physiological roles in the CNS, such as stimulation of glutamate release

(Clark et al., 1992), modulation of glutamate-mediated, long-term potentiation (Del Cerro et al., 1990; Arai and Lynch, 1992; Wieraszko et al., 1993; Bazan et al., 1993), and, when present at low concentrations, stimulation of PC12 cell sprouting (Kornecki and Ehrlich, 1988). When PAF synthesis is increased as a consequence of neuronal cell injury, it becomes a potent neurotoxin and contributes further to cell damage and death (Bazan, 1994). However, the mechanisms involved in PAF action are not well defined. PAF has the ability to stimulate early genes expression and some of these genes encode transcription factors involved in the expression of injury response genes (Bazan, 1994). Since the synthesis of specific nuclear proteins increases in neurons undergoing apoptosis (Villa et al., 1994), inhibition of RNA or protein synthesis protects neurons from apoptotic death (Martin et al., 1988). And, since light damage triggers photoreceptor apoptotic death (year 1 report), it is possible that PAF, by activating the expression of injury response genes, is a light damage injury mediator. The knowledge of the temporal sequence of signaling events triggered by light and their consequences for retinal function will help to define novel therapeutic targets that can successfully rescue photoreceptors from light damage.

BODY

Previous work. During the first year we followed at the light and EM level the time-sequence of events triggered by light damage in the retinal structure. We used two models of *in vivo* light damage: a long-term form (several days, normal light intensity) and a short-term form (two hours,7000 x lux). Use of both models reveals the sequence of events leading to delayed photoreceptor cell death. We found that

a) When animals are light-damaged during unrestrained, *in vivo* conditions, only a region located in the inferior-temporal retina, which receives maximal light, is damaged, while the inferior-nasal and superior areas af the retina are minimally affected.

b) The first structural change induced by light damage appears in the photoreceptor outer segment tips, moves slowly down the outer segment to the inner segment, and culminates as photoreceptors drop out and inner segments die.

c) Rod photoreceptors are more sensitive to light damage than are cones.

d) In the short-term model, two hours of excessive light will reduce the number of photoreceptors by 50% within two days, and leave only about 10-20% after one week.

e) Cell death results from light triggering the process of apoptosis. The pick of apoptosis, determined using the Ladder technique, occurs by 30 hours after the light insult, preceding cell drop out (maximal at 48 hours).

Objectives, year 2

1- To further explore at light level, using the TUNEL technique, the apoptotic death of photoreceptor cells following *in vivo* light damage (2 hours, 7000 lux).

2- Because the early intermidiary signals of light damage are generated during the exposure of animals for 2 hours to 7000 lux, and will lead to delayed photoreceptor death while animals are maintained at low intencity (2 lux) cycled light, we developed an *in vitro* model to induce light damage suitable for further identification of intermediary signals in isolated retinas.

This model has the following advantages: **a**) All photorecepors are exposed to the same light intensity and therefore the whole retina will be affected to a similar extent. While only one quarter of the retina (lower-temporal area) damaged after *in vivo* light exposure can be used for subsequent biochemical studies (i. e.looking at [^{3}H]DHA metabolism), the whole retina can be used when the *in vitro* protocol is followed. **b**) This model will allow direct access of potential blockers and inhibitors to photoreceptors, thus contributing to a precise biochemical and structural analysis of the subsequent events.

Material and Methods

Animals: Male, white Wistar rats (175-200 g body weight) purchased from Hill Top Laboratory Animals (Scottdale, PA), were kept in stainless steel, slotted topped cages and maintained in a 12 h light, 12 h dark photocycle, with ambient light levels set to about 2 lux. They were given water and rat chow *ad libitum*. Animals were mantained under these conditions for at least one week prior to the experiment.

In vivo short-term light damage: Rats were placed in white plastic cages and light damaged for 2 hours using a procedure developed during the first year of this proposal. Briefly, damage was initiated at 10:00 am using fluorescent light (24- inch bulbs, G. E.® cool white) supplying

approximately 7000 lux (125 $\mu E/m^2$ sec, or 7.5 x 10^{19} photons/m^2 sec). Animals were returned to their methal cages and kept under 2 lux ilumination for different periods of time.

In vitro light damage: On the day of each experiment, animals were maintained under dim light conditions (2 lux), anesthetized, and decapitated. Eyes were quickly removed, placed in cold buffer, and the anterior segments removed while maintaining the dim light condition. These eyecups were then placed on a platform over small openings within a chamber containing buffer, and the level adjusted to just cover the preparations. O_2:CO_2 (95%:5%) was continuously bubbled through a humidifier and the buffer under the platform by way of a small diameter, 10 port manifold. This chamber, in turn, was placed within a Dubnoff® constant temperature shaking water bath at 36° C. The chamber was not shaken because the constant movement of small bubbles through the buffer sufficiently agitated and mixed the preparation. Ames buffer, pH 7.3 was first gassed for 30 minutes and then added to the chamber. A bank of fluorescent lights (G.E.® cool white) was hung directly above the water bath, supplying 66 $\mu E/m^2$sec of continuous light throughout the experiments. Light intensity was measured prior to each experiment by placing the light sensitive surface of the light meter probe (LI-COR® quantum/radiometer/
photometer - Lincoln, NE) at the level of the retinal eyecups. Eyecups were then maintained under these conditions for up to 6 hours. Control eyecups were maintained in darkness.

Following the stimulation period, each eyecup was placed in cold fixative for a period of one hour and then cut in half, trimmed, and marked for orientation. They were then returned to fixative overnight, and then processed for embedding in plastic for electron microscopy or paraffin for light microscopy the next day.

Preparation of retinal tissue for histological analysis: Following light damage, animals were killed by over-anesthetization with ether or CO_2 exposure generated from dry ice. After eyes were removed and corneas slit, tissue was placed in fixative. The front of each eye was then removed, the lens lifted out, and the eyecup trimmed with a razor blade. Fixation for plastic-based light and electron microscopy followed conventional, published (Gordon and Bazan, 1990, 1993) procedures. Retinas were fixed with 2% glutaraldehyde / 2% formaldehyde in 0.135 M sodium cacodylate buffer (pH 7.3) overnight at 4° C, rinsed in cacodylate buffer (0.135 M) and

postfixed for 1 h in 1% OsO_4 in cacodylate buffer (0.135 M). When fixation had been completed, tissue was dehydrated and embedded in a mixture of Epon-araldite plastic (Mollenhauer, 1963), followed by sectioning. A diamond knife was used to cut silver-gold sections for electron microscopy, which were then placed on parlodion-coated, 100 mesh, hexagonal nickel grids and contrasted with uranium and lead salts.All retinal dissections and trimming were done with the aid of a Nikon SMZ-U stereo-zoom dissecting microscope. Ultra thin sections were viewed with a Zeiss C10 transmission electron microscope (Carl Zeiss, Jena, Germany), and photographed on 3¼" x 4" Kodak electron microscope film 4489 (EMS, Ft. Washington, PA). D-19 developer (Kodak, Rochester, NY) was used for 4 min.

Retinas were embedded in paraffin for TUNEL analysis according to published methods (Humason, 1972). Tissue was placed in 4% formaldehyde and phosphate buffered saline overnight, washed in phosphate buffered saline and dehydrated through an ethanol series to xylene. Tissue was then infiltrated and embedded in paraffin (56-57° C).

Metal knives were used to cut 5 μm-thick paraffin sections, which were then placed in distilled water on glass microscope slides (Superfrost/plus; Fisher Scientific, Pittsburgh, PA), and dried on a slide warmer. After deparaffinizing in xylene, sections were rehydrated and then processed for TUNEL analysis of 3'-nicked end DNA. Subsequently, sections were coverslipped with Aqua-Poly/Mount (Polysciences; Warrington, PA) and allowed to set. Counter staining was not employed.

Light microscope sections were viewed, analyzed, and photographed with a Nikon Optiphot-2 upright microscope and a Nikon UFX-DX automatic camera system which allowed 35mm photography of the sections.

Terminal dUTP Nick End Labeling (TUNEL) Technique: Generally, the technique described by Gavrieli et al. (1992) and Portera-Cailliau et al. (1994) was followed. Deparaffinized, rehydrated sections were treated with Proteinase K (20 μg/ml) in 10 mM TRIS-HCl (pH 8.0) for 15 min, and then rinsed four times (2 min each). Slides were then placed in 3% H_2O_2 for 5 min to inactivate endogenous peroxidases, and then rinsed three times in water. At this point, any positive controls were incubated with DNAse I (1 μg/ml) for 10 min at 37° C, and then washed. All slides were then preincubated for 10 min at room temperature in TdT buffer (30 mM TRIS-HCl,

140 mM sodium cacodylate, and 1 mM cobalt chloride; pH 7.2), followed by incubation for 1 h at 37° C in a moist chamber with 25-50 µl of TdT buffer containing 0.5 units/µl buffer and 40 µM biotinylated 16-dUTP. The reaction is stopped by immersing the slides in double strength SSC buffer (300 mM NaCl; 30 mM sodium citrate) for 15 min, and then rinsing in water and PBS buffer. Finally, sections were incubated for 1 h at 37° C in Vectastain® ABC peroxidase standard solution (Vector Laboratories; Burlingame, CA), rinsed twice in PBS, and stained for 30-60 min, using aminoethylcarbazole as a substrate. The reaction was stopped by rinsing in water, and sections coverslipped in Aqua-Poly/Mount (Polysciences; Warrington, PA). Apoptotic nuclei appear pink to purple in color, all others are colorless.

Ladder technique: The method of Tso et al. (1994) was used to recover DNA from experimental retinas. Frozen retinas were thawed in buffer consisting of Tris-HCl (10 mM, pH 7.6), Na_2EDTA (100 mM), SDS (0.5%), proteinase K (200 µg/ml), and RNAase A (50 µg/ml) for 3 h at 45 °C. The thick lysate was pushed through 19½-, 22½-, and 26½-guage needles, and the DNA extracted twice with phenol:chloroform:isoamylalcohol (25:24:1, by vol.) and once with chloroform. Following overnight precipitation at -20 °C in ethanol, DNA was dissolved in 200 µl of TE, containing Tris-HCl (10 mM, pH 7.6) and Na_2EDTA (1 mM). DNA concentration was determined by measuring its absorbance at 260 nm.

Portions of DNA samples (20 µg) were subjected to electrophoresis on a 1.8% agarose gel, containing ethidium bromide (0.5 µg/ml), in a running buffer of 1x TAE (pH 8.0) for 16 h at 35 volts. For calibrations, commercial, 123 DNA ladder samples were run along side. Gels were photographed under UV light.

Results

The region located in the inferior-temporal retina, which receives maximal light during unrestrained, *in vivo* light damage (7000 lux for 2 hours, first year report), was sectioned at 5 µm thickness, and the number and location of apoptotic (pink) photoreceptor nuclei observed in the light microscope by the Terminal dUTP Nick End Labeling (TUNEL) technique (Gavrieli et al., 1992; Portera-Cailliau et al. 1994). Pictures from retinal samples for negative controls (TdT enzyme omitted) and 24 hours post-damage are shown in **Figures 1A and 1B.** While control retinas (no light damage) showed no TUNEL labeling, within the region of light damage (the

inferior-temporal region), aproximtely 90% of the nuclei were labeled by 24 hours post- damage. A small population of cells appeared more heavily labeled than others, probably as a reflection of a more advanced stage in the apoptotic process and therefore more extensive DNA damage.

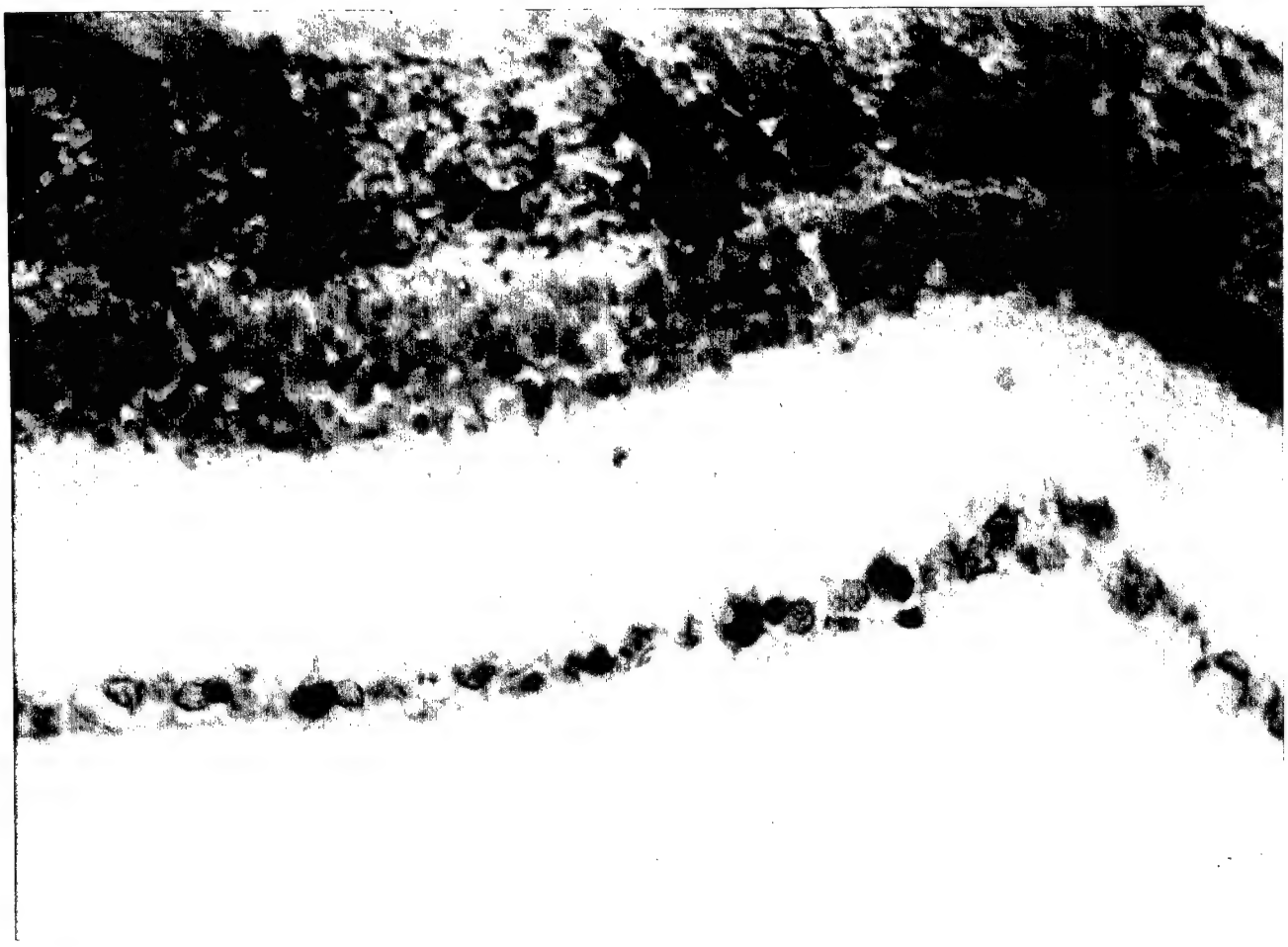

Figure 1A. Light micrograph of rat retina. 24 hours after a 2 hour exposure to 7000 lux fluorescent light, retinas were collected and prepared for paraffin sectioning and TUNEL labeling. Apoptotic nuclei of the photoreceptors are visible as dark red/brown spheres within the outer nuclear layer while, nuclei within deeper regions of the retina remain unlabeled, indicating that the light stimulus has triggered the process of cell death only within the photoreceptor cells. Several stages of apoptosis are visible. A few cells demonstrate overall darkening characteristic of advanced nuclear damage, but the majority of apoptotic photoreceptor nuclei show a dense ring at their periphery, indicating a more recent responce to light. Photoreceptor outersegments are up; retina/vitreal interface is down.

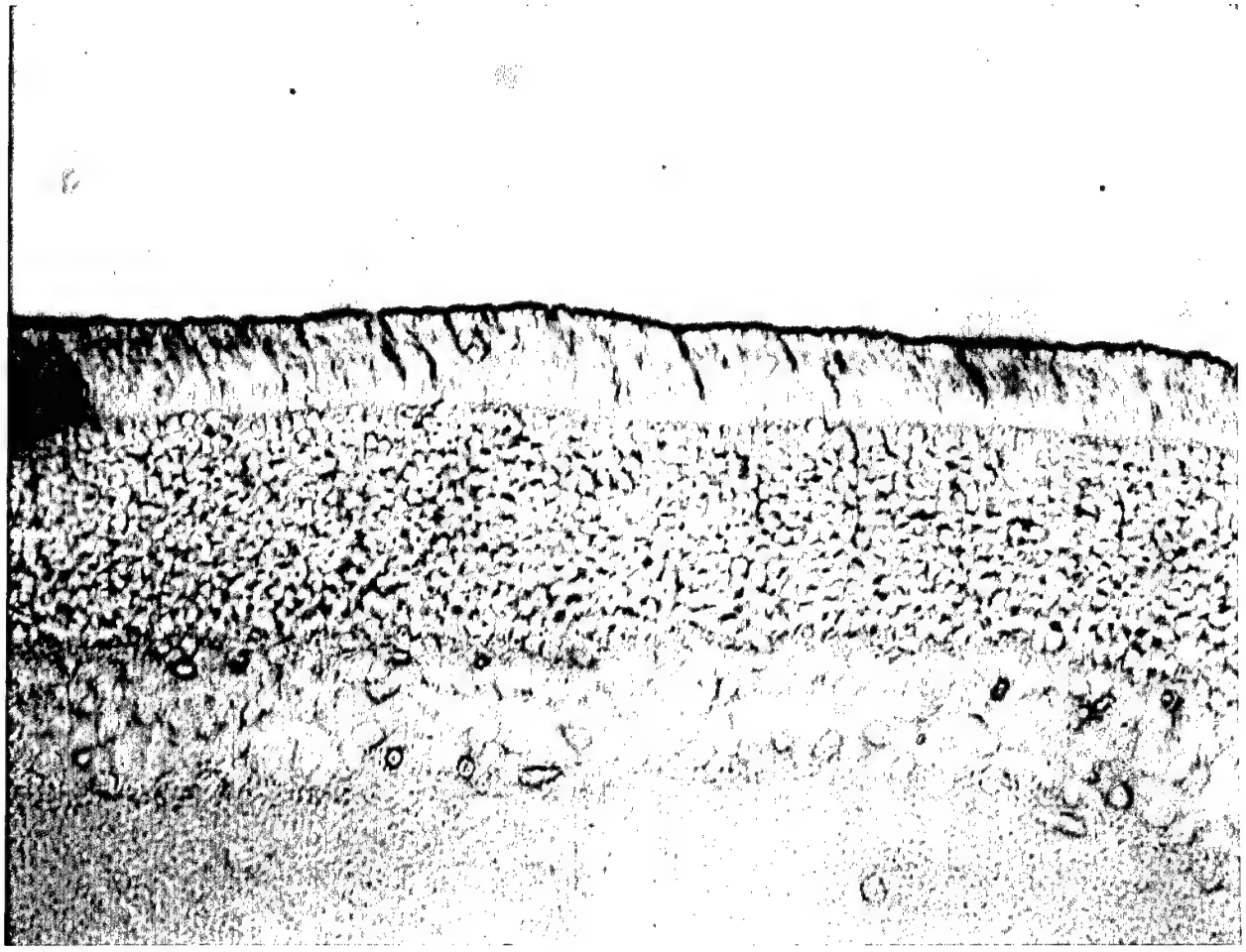

Figure 1B. Light micrograph of rat retina. This control section is from the same animal as shown in figure 1A. However, to demonstrate that this is not just nonspecific background, the labeled nucleotide (dUTP-digoxigenin) has been left out. Since no color appeared following the color reaction, all label seen in figure1A is due to labeled DNA. Photoreceptor outer segments are up; retina/vitreal interface is down.

After the *in vitro* light damage protocol (6 hours, 66 $\mu E/m^2$ sec), sections were prepared for light microscopy from randomly selected retinal areas. Using the TUNEL technique, pink apoptotic nuclei were detected after 6 hours of *in vitro* light damage in all the sections analyzed, while retinas incubated in the dark for similar period of time showed negative results. Pictures illustrating these results are shown in **Figure 2A and 2B**.

Figure 2A. Light micrograph of rat retina. This frozen section illustrates the presence of apoptotic photoreceptor nuclei in the retina, following 5 hours of stimulation by fluorescent light. The eyecup was immediately frozen and 5 μm-thick sections examined for positive TUNEL. Several apoptotic nuclei are indicated by the dense pink-red stain. Slight tissue distortions were caused during the sectioning procedure, not during stimulation. Photoreceptor inner and outer segments are up, whereas a split has occured just distal to the photoreceptor synaptic terminals at the bottom.

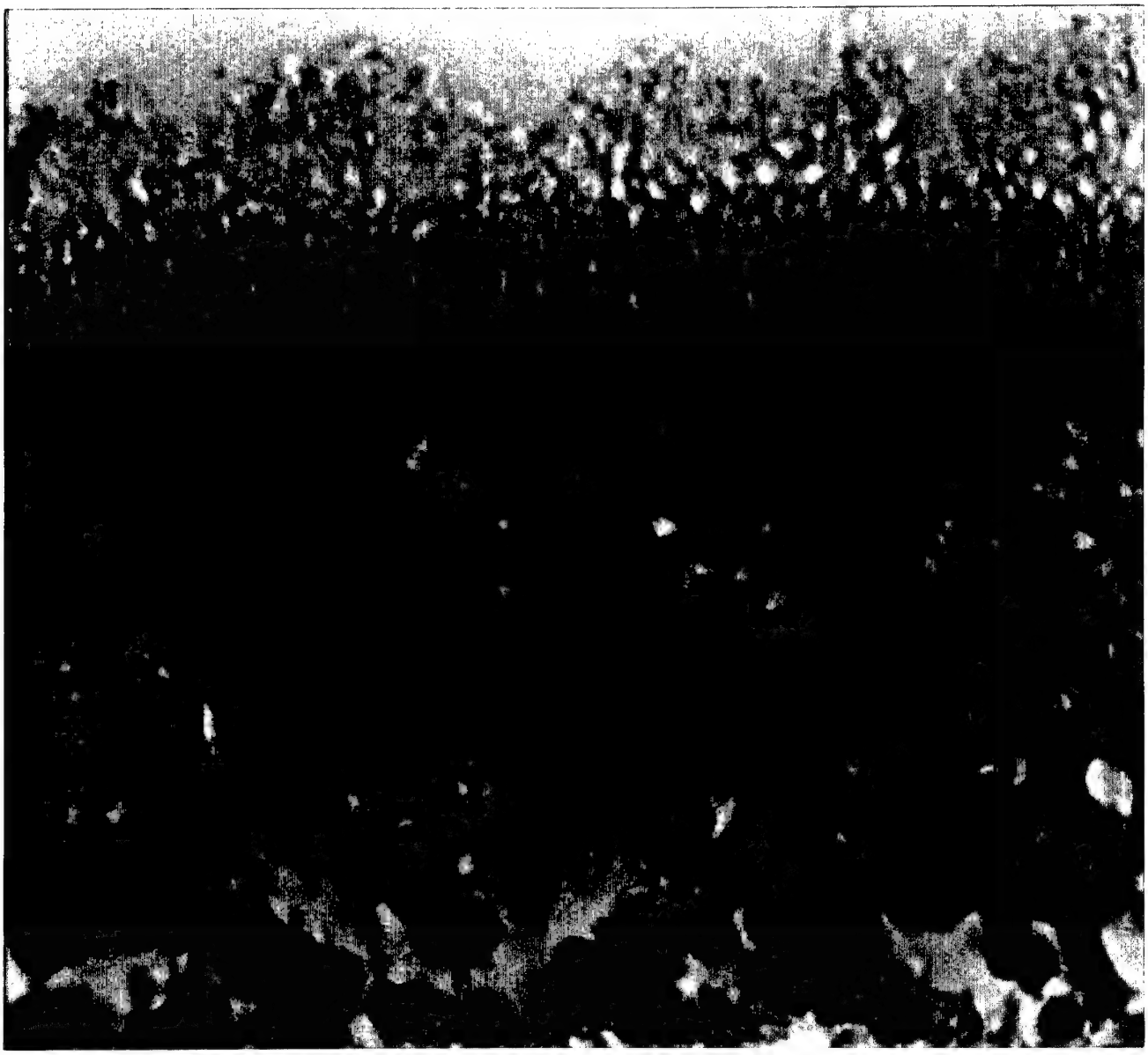

Figure 2B. Light micrograph of rat retina. Frozen 5 μm-thick section of a 5 hour dark incubated eyecup, examined by the TUNEL technique. No photoreceptor nuclei (central horizontal band) show signs of apoptosis. Photoreceptor inner and outer segments are located at the top, nuclei of the inner nuclear layer are present at the bottom.

Analysis of *in vitro* light damaged retinas by electron microscopy also confirms the presence of apoptotic nuclei. Some nuclei display peripherally condensed chromatin (**Figure 3**) while others showed darker apoptotic nuclei (**Figure 4**), an indication that cells are at different

stages in the apoptotic process.

When retinal samples were prepared for DNA laddering analysis, only weak banding appeared because of the relatively small number of apoptotic cells (data not shown).

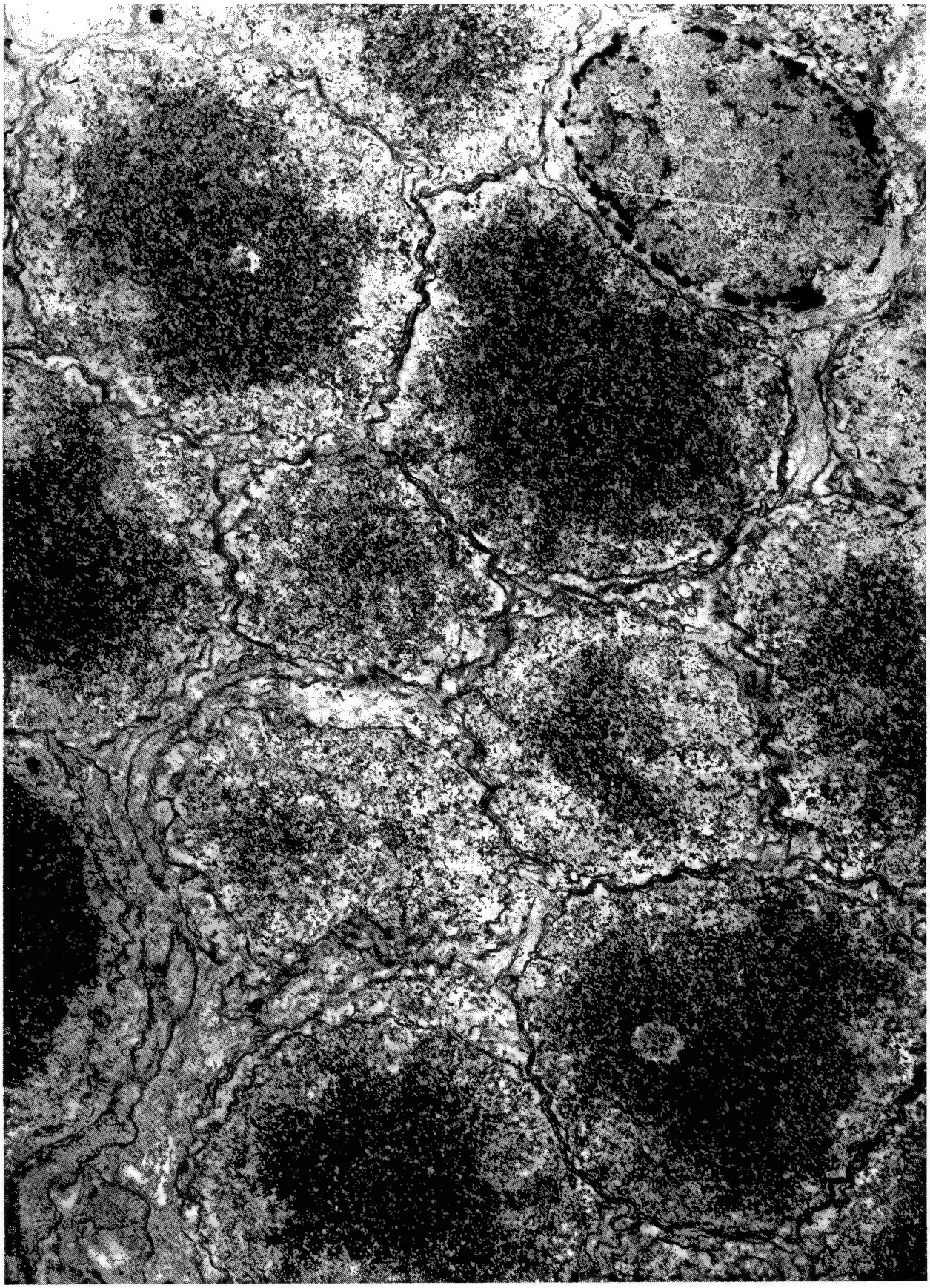

Figure 3.

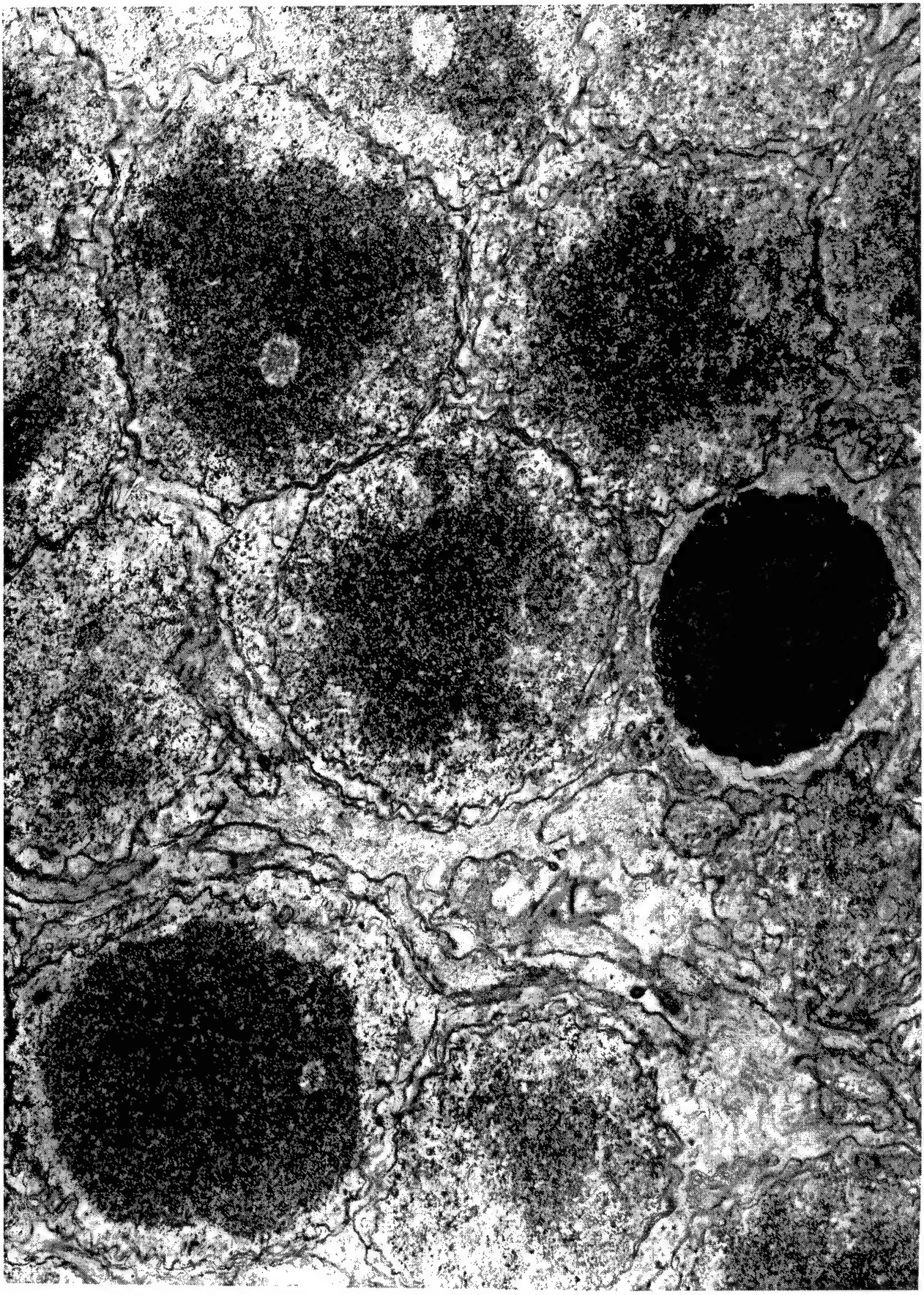

Figure 4.

SUMMARY AND CONCLUSIONS

In Vivo

- Apoptosis is already observed by TUNEL techniques at the light microscope level 20 hours after light exposure (2 hours, 7000 x Lux).
- Apoptotic nuclei coincide with the position of rod photoreceptor nuclei, not those of cones.

In Vitro

With our lighting regime, under *in vitro* conditions, apoptotic nuclei begin to appear within the outer nuclear layer by 6 hours.

- Electron microscopy demonstrates early apoptotic nuclei with peripherally condensed chromatin and older, darker apoptotic nuclei.
- TUNEL demonstrates apoptosis at the light microscope level.
- There is evidence of DNA laddering (data not shown), but only weak banding appears because of the relatively small number of apoptotic cells.
- The data suggest that the *in vitro* retina can serve as a model for the study of apoptosis.

Controlled bright light during retinal incubations can initiate apoptotic cell death, permitting studies of potential blockers and inhibitors, and allowing precise biochemical and structural descriptions of the subsequent events.

Research plan for the upcoming year

- Retinas will be exposed *in vitro* to brighter light for longer times (10-12 hours), to increase the number of apoptotic cells.
- The effect of the PAF antagonist BN50730 on light-induced photoreceptor apoptosis will be followed in both the *in vitro* and *in vivo* models.
- Retinal calcium mobilization will be analyzed in control and light-damaged retinas.
- [^{3}H]DHA metabolism will be followed in retinas incubated *in vitro* during and after both *in vivo* and *in vitro* light damage.

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